Laboratory Investigation (2013) 93, 825–833

The modest impact of transcription factor Nrf2 on the course of disease in an ALS animal model

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Oxidative stress is associated with the pathogenesis of amyotrophic lateral sclerosis (ALS). Nuclear factor erythroid 2-related factor 2 (Nrf2)–antioxidant response element (ARE) pathway is one of the major cellular defense mechanisms against oxidative stress. However, the role of Nrf2-mediated neuroprotection (antioxidant defense) in the disease development of ALS remains unclear. To further investigate the role of Nrf2 in ALS, we genetically eliminate the *Nrf2* gene from SOD1-G93A mice, a commonly used ALS mouse model, by generating a double mutant (Nrf2 − / − SOD1-G93A mice). We found that it only had a modest impact on the course of disease by knocking out *Nrf2* gene in these mice. Further studies demonstrated that, among previously known Nrf2-regulated phase II enzymes, only NAD(P)H: quinone oxidoreductase 1 induction was significantly affected by the elimination of *Nrf2* gene in SOD1-G93A mice. Taken together, our data suggested that Nrf2 is not the sole mediator for the induction of antioxidant genes in SOD1-G93A mice, and Nrf2-mediated neuroprotection is not the key protective mechanism against neurodegeneration in those mice.

Laboratory Investigation (2013) **93,** 825–833; doi:10.1038/labinvest.2013.73; published online 27 May 2013

KEYWORDS: amyotrophic lateral sclerosis (ALS); cytoprotective phase 2 enzyme; NF-E2-related factor 2 (Nrf2); oxidative stress; SOD1

Amyotrophic lateral sclerosis (ALS) is a progressively debilitating neurodegenerative disease, characterized by upper and lower motor neuron loss and eventually results in paralysis and death.[1] SOD1 mutations contribute to 20% of familial ALS, which is ∼5–10% of total ALS cases.[2] SOD1-G93A transgenic mice replicate most phenotypes of human ALS and are used widely in the pathogenetic and therapeutic studies.[3] It is well accepted that Oxidative stress contributes to the pathogenesis of ALS.[4–6] Nuclear factor erythroid 2-related factor 2 (Nrf2), a member of the 'cap 'n' collar' family of basic leucine zipper transcription factors,[7] binds to antioxidant response element (ARE) in the upstream region of its target genes promoting transcription of various cytoprotective genes, such as heme oxygenase-1 (HO-1),[8] NAD(P)H: quinone oxidoreductase 1 (NQO1),[9] and the catalytic subunit of glutamate cysteine ligase (GCLC),[10] which helps restore the oxidant–antioxidant balance in cells.[10,11] In this context, several studies demonstrated that Nrf2/ARE activators provided robust neuroprotective effect and ameliorated the disease progression in SOD1-G93A transgenic mice.[12,13] Notably, upregulation of Nrf2 expression was found in the nucleus of motor neurons in lumbar anterior horn of ALS model mice.[14] We also reported that significant HO-1 induction was detected in the lumbar spinal cord of ALS mice.[15] Overall, those findings support the notion that Nrf2 pathway-mediated neuroprotection might be important in protecting neurons against the insults of oxidative stress during the disease development of ALS.

To further evaluate the potential role of Nrf2 pathway played in the neurodegenerative process in ALS, we knocked out the *Nrf2* gene from SOD1-G93A mice by crossbreeding Nrf2-null mice with SOD1-G93A mice. Surprisingly, only modest deterioration on disease onset and life span was observed in Nrf2 − / − SOD1-G93A mice compared with Nrf2 + / + SOD1-G93A animal. The disease onset age was advanced by 7.5–10.5 days and the life span was shortened by 5.5–7.1 days in these double mutant animals (Nrf2 − / − SOD1-G93A). We found that only NQO1 induction was disabled in the target tissues and astrocytes of Nrf2 − / − SOD1-G93A mice, whereas the expression of HO-1 and GCL remains unchanged in these animals. In addition, the tissue level of GSH (glutathione), a major cellular antioxidant, was comparable between Nrf2 − / − SOD1-G93A mice and Nrf2 + / + SOD1-G93A animals. Overall, our data suggested

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Received 1 February 2013; revised 9 April 2013; accepted 24 April 2013

that Nrf2 has a limited role in preventing disease development and facilitating antioxidant defense in SOD1-G93A transgenic mice.

MATERIALS AND METHODS

Mice

The generation and genotyping of SOD1-G93A mice had been described previously.[3] Wild-type (Nrf2 + / +) and Nrf2-deficient (Nrf2 − / −) CD1/ICR mice were obtained from Dr Thomas W. Kensler (Johns Hopkins University, Baltimore, MD) and were genotyped using primers as follows: NRF5 (in *Nrf2* gene), 5′-TGGACGGGACTATTG AAGGCTG-3′; NAS (in *Nrf2* gene), 5′-GCCGCCT TTTCAGTAGATGGAGG-3′; and NLACZ (in *LacZ* gene), 5′-GCGGATTGACCGTAATGGGATAGG-3′. SOD1-G93A/Nrf2 + / + and SOD1-G93A/Nrf2 − / − mice were generated by mating SOD1-G93A transgenic male mice with Nrf2 + / + and Nrf2 − / − female mice, respectively, for 10 generations. The animals were housed in a temperature-, light-, and humidity-controlled environment and fed *at libitum* and drank water freely.

Animal Experiments

To monitor the disease progression, all animals were inspected daily for signs of motor deficit and weighed twice per week, starting at 12 weeks of age. Disease onset was determined when the following criteria were both met: (1) two continuous weight losses were observed after the animals reached their peak body weight; (2) earliest gait abnormalities were shown. The end stage was defined as the animals can no longer right themselves 30 s after being placed on their backs or sides.[16] The animals were killed at various time points (pre-symptomatic stage, disease onset, and end stage), and the tissues including spinal cord and cortex were collected thereafter. Animal experiments were carried out according to the regulations of laboratory animal management promulgated by the Ministry of Science and Technology of the People's Republic of China, which are in accordance with the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

Primary Astrocyte Culture

Primary astrocyte culture was established from brain cortex tissue of 1-day-old Nrf2 + / + or Nrf2 − / − mice, as described.[17] The dissociated astrocytes were kept in glial medium containing DMEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin (Amrisco), and 100 mg/ml streptomycin sulfate (Sigma). The culture media were changed twice a week. For the analysis of phase II enzymes induction, the astrocytes collected from Nrf2 + / + or Nrf2 − / − mice were treated with sulforaphane (SUL), at 10 μmol/l for 24 h.

Organotypic Spinal Cord Culture

Organotypic spinal cord culture was prepared as previously described.[18] Briefly, lumbar spinal cords were collected from 3-day-old mice pups under sterile conditions. The spinal cords were then sectioned transversely at 350-mm intervals using a tissue chopper (Mickle Laboratory Engineering, Surrey, UK). The cultures were maintained at 37 °C in a humidified incubator with 5% CO_2. One week after collection, the explants were treated with 10 μM SUL or vehicle control for 48 h at 37 °C, and then collected and stored at − 80 °C for further analysis.

Immunoblotting Analysis

The lysates were extracted from animal tissues, spinal cord explants, and primary astrocytes by using a total protein extraction kit (Applygen Technologies), according to the manufacturer's instruction. Bradford assay was conducted to determine the protein concentration of each sample. The lysates of each sample (50 μg) were run on 10% SDS-PAGE gel and transferred to PVDF membranes. The membranes were then probed with different primary antibodies, including anti-GCLC and anti-GCLM (Abcam, Cambridge, MA), anti-NQO1, anti-GSS, anti-keap1 and anti-SOD1 (Santa Cruz, CA), and anti-HO-1 (Stressgen Biotechnologies, Victoria, Canada). The bands of interest were detected using an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Band intensity was measured using the Odyssey Infrared Imaging System Version 2.1.12 on scanned infrared images. β-Actin or GAPDH was used as internal control. The original green or red color of a band was converted to black and white colors for data presentation.

Immunofluorescence Staining

The lumbar spinal cord was post-fixed in 4% paraformaldehyde for 24 h and then cryoprotected with 30% sucrose for 12 h. The tissues were sliced into 30 μm sections using a Leica CM1850 freezing microtome. The sections were washed for 3 times in PBS and then perforated with 0.3% Triton X-100. The tissue sections were double stained by anti-GFAP (Chemicon) and anti-NeuN (Santa Cruz) antibodies and detected with corresponding specific fluorophore-conjugated secondary antibodies. The nuclei were counterstained with Hoechst. Slides were mounted and analyzed by fluorescent confocal microscopy (Olympus FV1000).

Immunohistochemistry

The lumbar spinal cords were post-fixed in 4% paraformaldehyde, and cut into 20 μm sections using Leica VT 1000 S vibratome. The tissue sections were washed and perforated with 0.3% Triton X-100. After proper blocking, the tissues were incubated with anti-SMI-32 (Sternberger) and anti-GFAP (Chemicon) antibodies, overnight at 4 °C. The sections were then incubated with Biotin-conjugated secondary antibody (ZSGB-BIO, 1:200) for 2 h at room temperature (RT), followed by incubation with HRP-conjugated streptavidin (ZSGB-BIO, 1:200) for 1 h. The staining was visualized by treating the sections with 0.03%

diaminobenzidine for 10 min at RT. Slides were mounted and analyzed by light microscopy (Olympus BX51).

The numbers of motor neurons (SMI-32 positive) with intact nucleus, $\geq 25\,\mu$m in diameter and astrocytes (GFAP positive) with intact cell bodies in the anterior horn of lumbar spinal cord were counted.

Glutathione Assay

Lumbar spinal cords were dissected freshly and snap-frozen in liquid nitrogen. The tissue was then ground in deproteinizing agent and the suspension was centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was measured for GSH content using a GSH assay kit, according to the manufacturer's instruction (Beyotime Inst Biotech, Jiangsu, China).

Statistical Analysis

All data are presented as mean ± s.d. Ages of onset and survival rates were analyzed using Kaplan–Meier analysis. Differences among groups were determined using one-way ANOVA followed by SNK multiple-range test. $P<0.05$ was considered statistically significant. All statistical analysis was conducted using SPSS 16.0 software.

RESULTS

Significant Upregulation of Cytoprotective Phase II Enzymes in the Lumbar Spinal Cord of Sod1-G93a Mutant Mice

Oxidative damage is involved in the pathogenesis of ALS. Nrf2 is widely believed to have a critical role in cellular defense against oxidative stress and other toxicities by transcriptionally simulating a wide array of antioxidant proteins and detoxification enzymes via ARE in the target genes. We first examined the expression of several well-known antioxidant genes, including HO-1, NQO1, GCL (both the catalytic subunit GCLC and the regulatory subunit GCLM), and GSS, in lumbar spinal cord and motor cortex of SOD1-G93A transgenic mice at various disease stages. Significant upregulation of HO-1, NQO1, GCLC, and GCLM were detected in the lumbar spinal cord of SOD1-G93A mice at the disease onset and end stages, whereas no significant changes in the expression levels of these proteins were observed in motor cortex of SOD1-G93A mice at either stage (Figure 1a). Quantitative analysis showed 1.5–3.5 fold increases in protein expression of HO-1, NQO1, GCLC, and GCLM were found in the lumbar spinal cord of SOD1-G93A mice, compared with non-transgenic littermates (Figure 1b). Our data suggested that there is severe oxidative stress in the lumbar spinal cord of SOD1-G93A mice, while the motor cortex of those mice is relatively spared.

The Modest Impact of *Nrf2* Gene Knockout on Disease Progression in Sod1-G93a Mutant Mice

Nrf2 activators were shown to be neuroprotective and able to slow disease progression in a mouse model of ALS.[12] To further study the involvement of Nrf2 pathway in the process

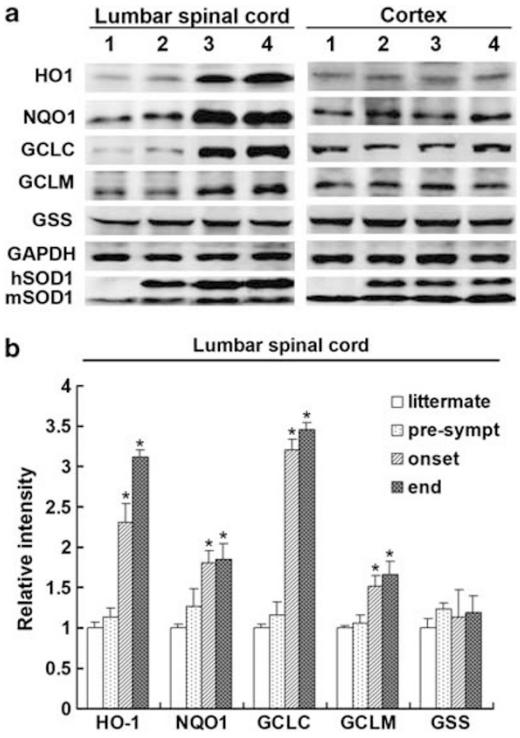

Figure 1 Induction of phase II enzymes in the lumbar spinal cord of SOD1-G93A mice. (**a**) Immunoblotting images showing the expressions of phase II enzymes (heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), catalytic subunit of glutamate cysteine ligase (GCLC), GCLM, and GSS) in the lumbar spinal cord and motor cortex of SOD1-G93A mice at different disease stages. 1, littermate; 2, pre-symptomatic stage (60 days of age); 3, onset stage; and 4, end stage. (**b**) Quantitative densitometry showing the expression of phase II enzymes in the lumbar spinal cord. Three females and three males were examined at each stage. Data were obtained from three independent experiments. *$P<0.05$, compared with littermate.

of motor neuron degeneration in SOD1-G93A mice, we generated double mutant mice, Nrf2 −/− SOD1-G93A, by mating SOD1-G93A mice to Nrf2 −/− animals with the same genetic background. The *Nrf2* gene knockout was confirmed by PCR examination (Figure 2a). As shown in Figures 2b and c, SOD1-G93A/Nrf2 −/− mice exhibited earlier disease onset and shorter life span, compared with SOD1-G93A/Nrf2 +/+ mice. The disease onset age of SOD1-G93A/Nrf2 −/− mice was 88.0 ± 7.7 days (male) and 95.4 ± 4.9 days (female), compared with 98.5 ± 3.2 days and 102.9 ± 5.1 days for male and female SOD1-G93A/Nrf2 +/+ mice, respectively. The average life span of SOD1-G93A/Nrf2 −/− mice was 108.5 ± 8.5 days for male and 115.9 ± 9.9 days for female, compared with 114.0 ± 7.6 days and 123.0 ± 9.8 days for male and female SOD1-G93A/Nrf2 +/+ mice, respectively (Table 1). Despite of the earlier disease onset and shorter life span observed in SOD1-G93A/Nrf2 −/− mice, we found similar motor neuron loss and astrocytosis in the anterior horn of lumbar spinal cord at the end stage between SOD1-G93A/Nrf2 +/+ mice and SOD1-G93A/Nrf2 −/− mice (Figure 2d).

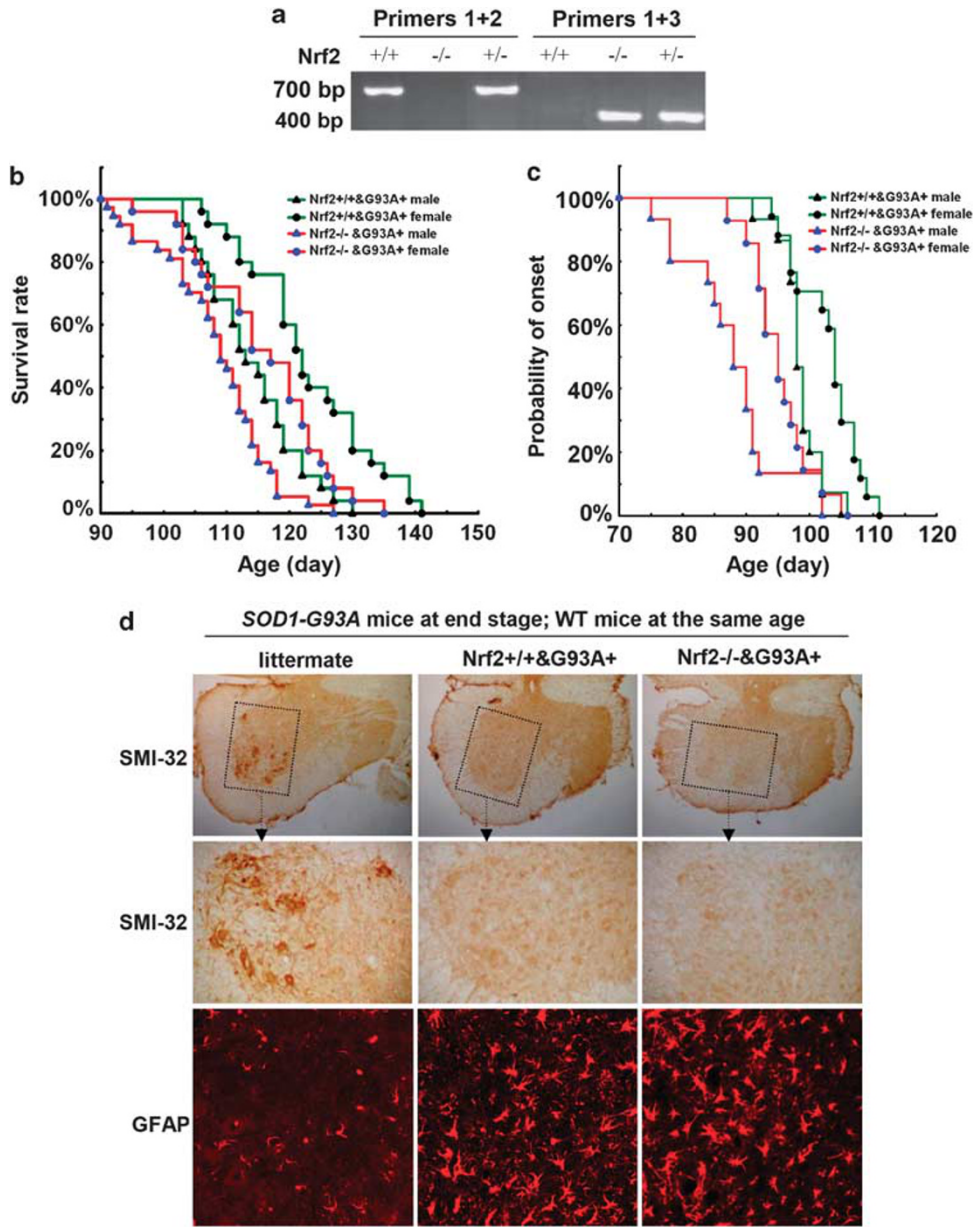

Figure 2 Effect of nuclear factor erythroid 2-related factor 2 (Nrf2) knockout on disease progression of SOD1-G93A mice. (**a**) PCR identification of Nrf2 knockout mice. (**b**) Survival rate of Nrf2 + / + SOD1-G93A and Nrf2 − / − SOD1-G93A mice. (**c**) Probability of disease onset of Nrf2 + / + SOD1-G93A and Nrf2 − / − SOD1-G93A mice. (**d**) Immunohistochemical analysis of motor neuron loss and immunofluorescence analysis of astrocytic proliferation in the lumbar spinal cord of Nrf2 + / + SOD1-G93A and Nrf2 − / − SOD1-G93A mice. SMI-32 and GFAP were used as markers for motor neuron and astrocytes, respectively. Data were representative of three females and three males in each group.

The Effect of Nrf2 Knockout on Motor Neuron Survival and Astrocytic Proliferation

ALS is pathologically characterized by motor neuron loss, and activated astrocytes have an vital role in the induction and propagation of motor neuron loss.[19] To examine the pathological changes in the lumbar spinal cord of SOD1-G93A mice after Nrf2 knockout, SMI-32 and GFAP immunohistochemistry were performed on the lumbar spinal cord tissues collected from SOD1-G93A/Nrf2 − / − and SOD1-G93A/Nrf2 + / + mice at various stages (pre-symptomatic and onset stage). Significant motor neuron loss was observed in SOD1-G93A/Nrf2 − / − mice at pre-symptomatic stage, whereas neuronal loss with similar extent was not detected till onset stage in SOD1-G93A/Nrf2 + / + mice. Astrocytic proliferation was significant at 60 days of age and became more prominent at 88–95 days of age in both SOD1-G93A/Nrf2 + / + and SOD1-G93A/Nrf2 − / − mice. However, there were more proliferating astrocytes found in SOD1-G93A/Nrf2 − / − mice compared with SOD1-G93A/Nrf2 + / + animals (Figure 3).

Table 1 Life span and age of onset of Nrf2 + / + &G93A/F2 and Nrf2 − / − &G93A/F2 mice

	Life span		Age of onset	
Group	Male	Female	Male	Female
Nrf2 + / + &G93A + /F2 (number of mice)	114.0 ± 7.6 (25)	123.0 ± 9.8 (25)	98.5 ± 3.2 (15)	102.9 ± 5.1 (17)
Nrf2 − / − &G93A + /F2 (number of mice)	108.5 ± 8.5* (37)	115.9 ± 9.9* (25)	88.0 ± 7.7* (15)	95.4 ± 4.9* (14)

Wild-type (Nrf2 + / +) and Nrf2-deficient (Nrf2 − / −) CD1/ICR female mice were crossed to B6SJL-Tg (SOD1-G93A) 1Gur/J male mice. SOD1-G93A transgenic male mice in the F1 generation were backcrossed to Nrf2 + / + and Nrf2 − / − CD1/ICR female mice. In the F2 generation, Nrf2 + / + and SOD1-G93A transgenic mice (SOD1-G93A/Nrf2 + / + /F2), and Nrf2 − / − and SOD1-G93A transgenic mice (SOD1-G93A/Nrf2 − / − /F2) were generated. 43 Nrf2 + / + &G93A/F2 mice from 14 litters and 58 SOD1-G93A/Nrf2 − / − /F2 mice from 31 litters were analyzed for life span. Thirty-two SOD1-G93A/Nrf2 + / + /F2 mice from 12 litters and 29 SOD1-G93A/Nrf2 − / − /F2 mice from 20 litters were analyzed for age of disease onset. *$P<0.05$, compared with SOD1-G93A/Nrf2 + / + /F2 mice of the same sex.

The Effect of Nrf2 Knockout on the Induction of Phase II Enzymes in Sod1-G93a Mice

Activation of Nrf2-ARE pathway has been shown to be protective against oxidative stress-induced cell death.[20] Several lines of evidences have suggested that the induction of Nrf2-regulated phase II enzymes, including HO-1, NQO1, and GCLM, etc, had strongly protective effects against oxidative damage and neuronal death.[18,21] In addition, it was reported that GCLM knockout decreased the life span of SOD1-G93A mice by 55%, which was related to the reduction of GSH.[22] To better understand the role of Nrf2 in ALS, we examined the expression of several phase II enzymes in different tissues, including lumbar spinal cord and motor cortex, of Nrf2 + / + and Nrf2 − / − SOD1-G93A mice (Figure 4). Among the five enzymes examined (HO-1, NQO1, GCLC, GCLM, and GSS), the expressions of four enzymes were significantly increased (1.8–4.3 fold) in the lumbar spinal cord of Nrf2 + / + SOD1-G93A mice compared with that of non-transgenic Nrf2 + / + littermates, except for GSS. Interestingly, three of the examined enzymes, including HO-1, GCLC, and GCLM, were significantly induced (2.3–4.2 fold) in Nrf2 − / − SOD1-G93A mice as well, compared with the Nrf2 − / − littermates. Further analysis showed that the induction of NQO1 in lumbar spinal cord of SOD1-G93A/Nrf2 + / + mice was significantly higher than that of SOD1-G93A/Nrf2 − / − mice. However, the expressions of four other examined phase II enzymes in lumbar spinal cord remain unchanged after Nrf2 knockout in SOD1-G93A mice (Figure 4). No obvious motor neuron loss and expression changes of HO-1, NQO1, GCLC, GCLM, and GSS were observed in the motor cortex of Nrf2 − / − SOD1-G93A mice compared with Nrf2 + / + SOD1-G93A mice (Supplementary Figure 1).

As Keap1 sequesters Nrf2 in the cytoplasm and is an important modulator of Nrf2/ARE pathway, we further examined the expression of keap1. We found significant upregulation of keap1 in both Nrf2 + / + and Nrf2 − / − SOD1-G93A mice at end stage compared with non-transgenic littermates, while the former showed much higher keap1 induction than the latter (Figures 4c and d).

GSH has a key role in cellular redox signaling and protecting against oxidative injury,[23] thereby, we examined the GSH levels in different tissues of Nrf2 + / + and Nrf2 − / − mice. Interestingly, there was significantly less GSH in the liver, bladder, and lung of Nrf2 − / − mice, compared with Nrf2 + / + mice. However, GSH content in lumbar spinal cord, motor cortex, and kidney was comparable between Nrf2 − / − and Nrf2 + / + mice (Figures 5a and b).

Role of Nrf2 in Phase II Enzymes Induction in Cultured Primary Astrocytes and Spinal Cord Explants

The role of Nrf2 in phase II enzymes induction was further studied in *in-vitro* models. Astrocytes are the major reactive glial cells companying motor neuron loss in SOD1-G93A transgenic mice.[24] In the current study, we also examined the effect of Nrf2 on the induction of phase II enzymes in astrocytes. Primary astrocytes isolated from the brain cortex of Nrf2 + / + and Nrf2 − / − mice were treated with Nrf2-ARE activator, SUL. SUL induced robust upregulation of HO-1, NQO1, and GCLM in Nrf2 + / + astrocytes, and it also significantly upregulate the expression of HO-1 in Nrf2 − / − cells. However, the SUL-induced upregulations of NQO1 and GCLM were abolished in Nrf2 − / − astrocytes. The GCLC and GSS expressions were not altered in either type of astrocytes by the treatment of SUL, although Nrf2 − / − cells expressed somewhat lower basal level of GSS than Nrf2 + / + cells (Figures 6a and b). These data suggested that Nrf2 pathway is associated with NQO1 and GCLM induction in astrocyte, but it might not regulate the expression of HO-1, GCLC, and GSS in those cells. The Nrf2-independent HO-1 and Nrf2-dependent NQO1 induction in astrocytes by SUL are in compliance with our *in-vivo* findings (Figure 4). Similar studies were also conducted on spinal cord explants. We found that SUL induced significant upregulation of NQO1 in an Nrf2-dependent manner, whereas the expression of GCLC was not altered by the SUL treatment and no difference was observed between Nrf2 + / + mice and Nrf2 − / − mice (Figure 6c).

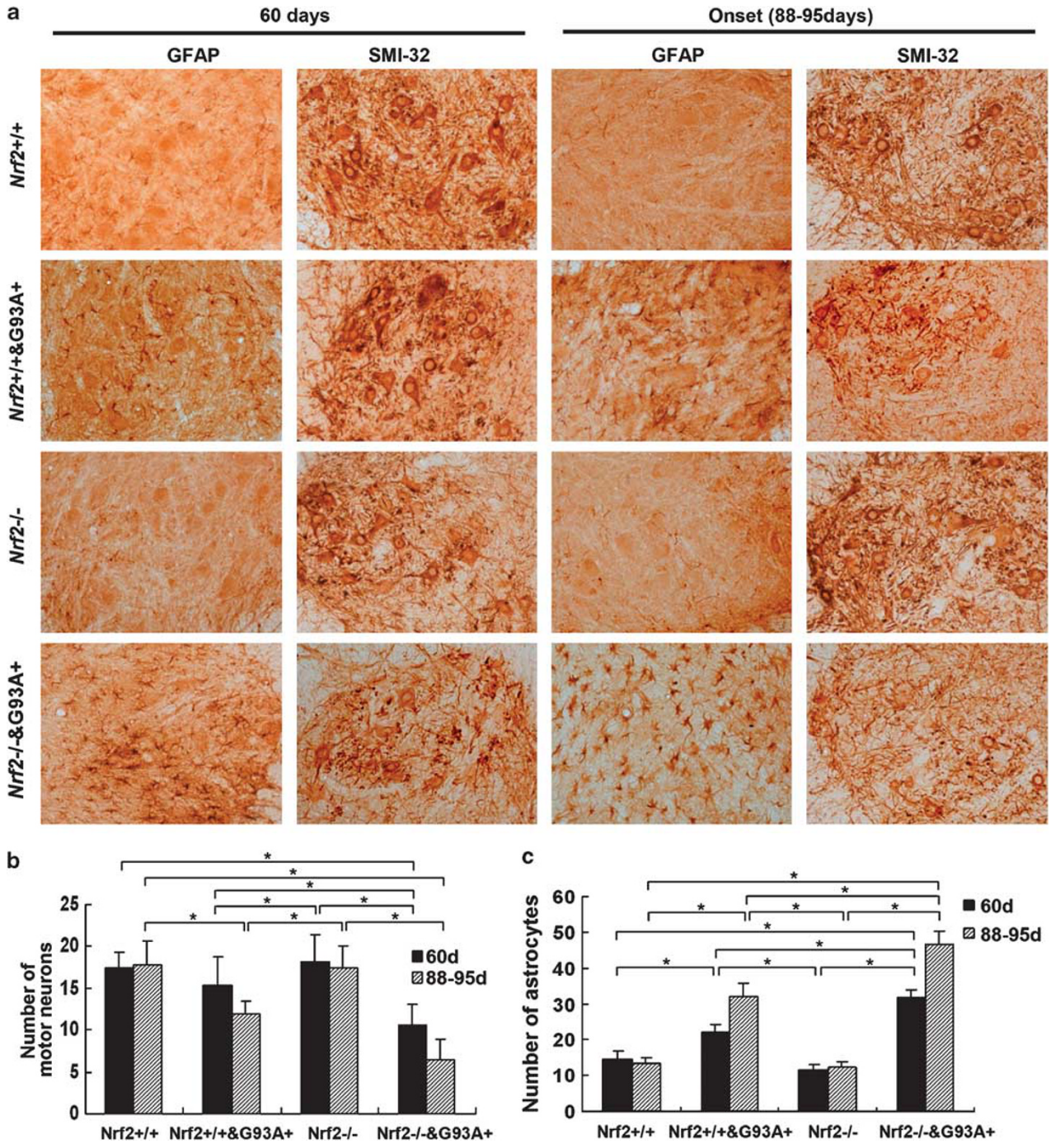

Figure 3 Earlier motor neuron loss and astrocytic proliferation in nuclear factor erythroid 2-related factor 2 (Nrf2) − / − SOD1-G93A mice. (**a**) Micrographs showing motor neurons (SMI-32) and astrocytes (GFAP) in the lumbar spinal cord of Nrf2 + / + SOD1-G93A, Nrf2 − / − SOD1-G93A mice and their littermates at 60 and 88–95 days of age. (**b**) Quantitative analysis of motor neuron numbers. (**c**) Quantitative analysis of astrocyte numbers. $^*P < 0.05$. Data are representative of three males in each group.

DISCUSSION

Nrf2-mediated regulation of cellular antioxidant production has an important role in neuroprotection against some neurodegenerative diseases. Activation of Nrf2 pathway promotes the transcription of various cytoprotective genes, including *HO-1*, *NQO1*, and *GCLC*, etc. HO-1 is highly inducible by various pro-oxidant and other stressors.[25] Upregulated HO-1 expression has been found in the brain of patients with AD,[26] and in the lumbar spinal cord of SOD1-G93A transgenic mice.[15] NQO1, a redox-regulated flavoenzyme, is sensitive in monitoring cellular redox state.[27] GCLC, GCLM, and GSS are enzymes essential for synthesis of

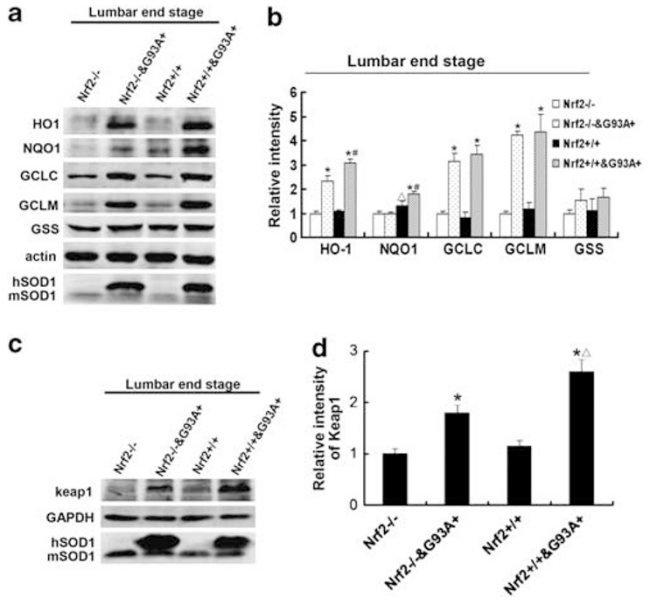

Figure 4 Phase II enzyme inductions and keap1 expression in the spinal cord of nuclear factor erythroid 2-related factor 2 (Nrf2) + / + SOD1-G93A and Nrf2 − / − SOD1-G93A mice. (**a**) Immunoblot analysis of phase II enzymes. Inductions of heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), catalytic subunit of glutamate cysteine ligase (GCLC), GCLM, and GSS in the lumbar spinal cord were compared between Nrf2 + / + SOD1-G93A and Nrf2 − / − SOD1-G93A mice at end stage. Data were representative of three females and three males in each group. (**b**) Histograms of phase II enzyme expressions in **a**. $^{*}P<0.05$, compared with non-transgenic littermate; $\triangle P<0.05$, compared with Nrf2 − / − mice; and $^{\#}P<0.05$, compared with Nrf2 − / − SOD1-G93A mice. (**c**) Changes of keap1 expression in the lumbar spinal cord of Nrf2 + / + SOD1-G93A and Nrf2 − / − SOD1-G93A mice at end stage. Data were representative of three independent experiments. (**d**) Histograms of keap1 expression in **c**. $^{*}P<0.05$, compared with non-transgenic littermate; $\triangle P<0.05$, compared with Nrf2 − / − SOD1-G93A mice.

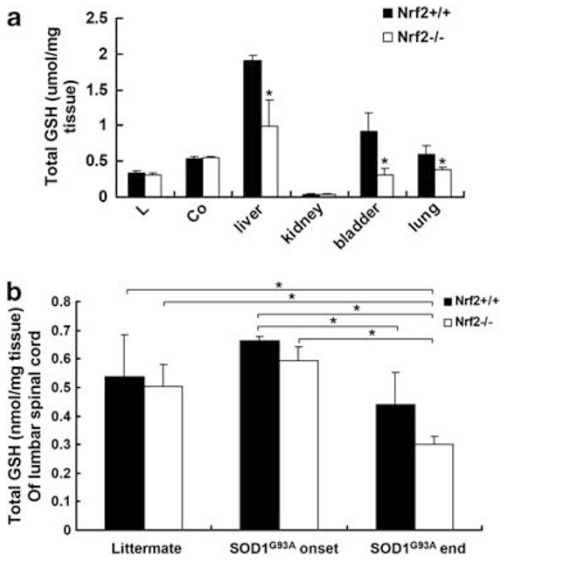

Figure 5 Changes of glutathione (GSH) content in the spinal cord of nuclear factor erythroid 2-related factor 2 (Nrf2) + / + SOD1-G93A and Nrf2 − / − SOD1-G93A mice. (**a**) Total GSH content in different tissues of Nrf2 + / + and Nrf2 − / − mice. $^{*}P<0.05$, compared with the same tissue of Nrf2 + / + mice. Data are representative of three females at 90 days of age. (**b**) Total GSH content in the lumbar spinal cord of non-transgenic littermates (two females and two males), Nrf2 + / + SOD1-G93A, Nrf2 − / − SOD1-G93A mice at onset (one female and two males) and end stages (two females and one male). $^{*}P<0.05$.

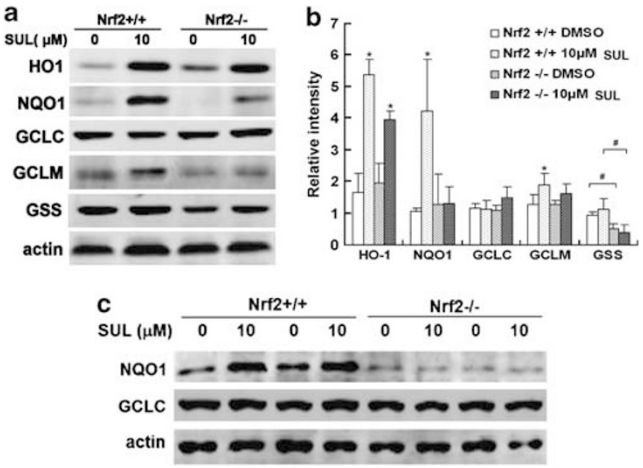

Figure 6 Induction of phase II enzymes in cultured astrocytes and spinal cord explants treated with sulforaphane (SUL). (**a**) Immunoblot analysis of phase II enzyme inductions in nuclear factor erythroid 2-related factor 2 (Nrf2) + / + and Nrf2 − / − astrocytes treated with 10 μM SUL for 24 h. Data are representative of three independent experiments. (**b**) Histograms of phase II enzyme expressions in **a**. $^{*}P<0.05$, compared with untreated group. $^{\#}P<0.05$, compared with Nrf2 + / + group with or without 10 μM SUL treatment. (**c**) NAD(P)H: quinone oxidoreductase 1 (NQO1) was induced by SUL in the spinal cord explants from Nrf2 + / + and Nrf2 + / + SOD1-G93A mice, but not Nrf2 − / − and Nrf2 − / − SOD1-G93A mice. Total extracts of lumbar spinal cord explants were examined for the expression levels of NQO1 and catalytic subunit of glutamate cysteine ligase (GCLC) by western blotting. β-Actin was used as a control. The results are representative of triplicate experiments. HO-1, heme oxygenase-1.

GSH, which is the major endogenous thiol antioxidant, counteracting oxidative injury within cells.[23] Upregulation of HO-1, NQO1, GCLC, and GCLM in the lumbar spinal cord of SOD1-G93A mice implicates that the affected tissues are experiencing chronic oxidative stress. Although the expression of Nrf2-mediated antioxidant genes confers cytoprotection, longer-term induction of some enzymes such as HO-1 may exacerbate oxidative injury to the mitochondria and the microtubular network.[28]

Nrf2 and its downstream ARE-driven genes have a very important role in protecting cells from the insults of oxidative stress.[29] Several studies demonstrated that Nrf2 knockout deteriorate the oxidative damage under certain circumstance.[30,31] However, in the current study, only modest impacts on disease progression were found with the absence of Nrf2 activity in SOD1-G93A mice. The induction of ARE-driven genes is required for the Nrf2-mediated neuroprotection, and the knockout of some ARE-driven genes, such as CCS and GCLM, had dramatic effects (significantly decreased survival) in ALS animal model.[22,32]

We found that NQO1 induction was disabled by Nrf2 knockout, whereas, the induction of HO-1, GCLC, and GCLM does not differ between Nrf2 − / − SOD1-G93A and Nrf2 + / + SOD1-G93A mice. Consequently, the GSH content in lumbar spinal cord was not altered in Nrf2 − / − mice compared with controls. The modest impacts on GSH level and the induction of ARE-driven genes by Nrf2 knockout explain the mild change in the course of disease found in Nrf2 − / − SOD1-G93A mice. SUL is a well-known chemopreventive phytochemical, capable of activating Nrf2-regulated cytoprotective signaling pathway.[33] Our results showed that SUL induced upregulation of NQO1 in an Nrf2-dependent manner, however, Nrf2 activation by SUL does not lead to induction of GCLC and GSS, even though GCLC gene promoter is known to carry a functional ARE. Similar modulation of these phase II enzymes was observed during keratinocyte differentiation as well.[34] These results suggested that other mechanisms besides Nrf2 pathway might be involved in the induction of the antioxidant genes in this ALS mouse model. Indeed, previous study showing the induction of HO-1 in Nrf2-deficient MEF and HEK-293 cells provided evidence that HO-1 can be regulated by Nrf2-independent mechanisms.[35] Moreover, it has been reported that Nrf1, another member of CNC family transcription factors, may compensate for the deficiency of Nrf2.[36,37]

The breeding strategy we used by inbreeding SOD1-G93A/Nrf2 + / + and SOD1-G93A/Nrf2 − / − mice separately without mixing may contribute to the moderate difference in disease progression. Furthermore, the main non-neuronal cells in the central nervous system are astrocytes, which contribute to the maintenance of normal neuronal function. Astrocytes isolated from SOD1-G93A mice are toxic to cocultured motor neurons.[38] On the other hand, a recent study suggested that Nrf2 overexpression in astrocytes significantly delayed onset and extended survival of SOD1-G93A mice.[39] GST secretion by astrocytes has been demonstrated to be protective for neurons against oxidative stress.[40] The *Nrf2* gene was genetically depleted from both neurons and astrocytes in the double mutant animals generated in our current study, and the depletion of Nrf2 in the proliferated astrocytes can exacerbate the toxicity to motor neurons and contribute to the deteriorative motor neuron loss in Nrf2 − / − SOD1-G93A mice.

Similar phenomena were observed in cultured spinal cord slices. Anyhow, different cell types under different settings may contribute to the *in-vivo* and *in-vitro* differences in phase II enzyme induction.

We demonstrated here that there was marked induction of phase II enzymes in the lumbar spinal cord of SOD1-G93A mice compared with non-transgenic littermates, while no induction was observed in motor cortex. Nrf2 knockout led to accelerated motor neuron loss and astrocytic proliferation, and caused earlier disease onset and shorter life span in SOD1-G93A transgenic mice. However, the effect of Nrf2 knockout is relatively modest. Our data show that the induction of antioxidative genes, except *NQO1*, such as *HO-1* and *GCL*, and the tissue GSH level are independent of Nrf2 activity, which may contribute at least in part to the limited effect of Nrf2 knockout on this ALS animal model. Taken together, our findings suggest that Nrf2 is not the key mediator regulating protections against SOD1 mutant-induced chronic neurodegeneration in this ALS animal model, and further investigations are granted to study the precise neuroprotective mechanisms in ALS and determine the potential targets for designing more effective therapeutic approach.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGEMENTS

We thank Yongbo Sui for his technical supports and Dr Thomas W. Kensler (John Hopkins University, Baltimore, MD, USA) for providing us the breeders of Nrf2-deficient mice. This work was supported by the Natural Science Foundation of China (grant numbers 30900460 and 81171210) and the Science and Technological Department of Hebei Province (grant number 11966122D).

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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